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Use of Unmodified Starches and Partial Removal of Serum To Improve Granada Medium Stability

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The use of 1% unmodified rice starch and 1% horse serum instead of 2% soluble starch and 5% serum in Granada medium is described. These components result in a medium of increased stability, preventing spoilage after a few days of storage at room temperature.

The detection of orange-red colonies in Granada medium (GM) (5) is an easy way of identifying hemolytic *Streptococcus agalactiae* (a group B streptococcus [GBS]) in clinical samples (1, 3, 9, 11, 15, 17, 20). Proteose Peptone 3 (BD/Difco, Franklin Lakes, N.J.) (12, 18), soluble starch (6), and horse serum (10, 13) are necessary components of culture media aimed to detect GBS by pigment production. In addition to these components, GM contains a folate pathway inhibitor (methotrexate) (4, 5), a Good's buffer (MOPS [morpholinepropanesulfonic acid]) (5, 8), and glucose (5) to trigger GBS pigment production.

However, an important drawback of GM is its poor stability if not stored refrigerated. The use of improperly stored GM can cause failure in the detection of GBS (7, 14, 20). We hypothesized that hydrolysis of soluble starch caused by the amylase in the serum (16) may be an important factor in the spoilage of nonrefrigerated GM (M. De La Rosa-Fraile, Letter, J. Clin. Microbiol. **41:**4007, 2003). Here we report on the use of unmodified starch, instead of soluble starch, and the reduction or removal of the serum in order to increase the stability of nonrefrigerated GM.

An overnight culture of GBS strain ATCC 12386 in brain heart broth diluted 1/100 in 0.85% NaCl was used for initial testing. The GBS pigment was graded 0 (no pigment), 1+ (yellow), 2+ (pale orange), 3+ (orange-red), or 4+ (deep red). Colony size and pigment score were assessed after 18 h of anaerobic incubation at 36°C.

Several formulations of GM were prepared with 1 or 2% soluble starch (catalog no. 1252; Merck HGaA, Darmstadt, Germany) and the following unmodified starches (from Sigma-Aldrich Corp., St. Louis, Mo.): cornstarch (catalog no. S 4126), rice starch (catalog no. S 7260), and wheat starch (catalog no. S 5127). Each medium was prepared with 5, 2, or 1% horse serum or without horse serum. Plates were inoculated as they were prepared or after storage for 6 days at 4, 22, or 30°C.

When GM plates were inoculated with GBS strain ATCC 12386, either as they were prepared or after 6 days of storage in the refrigerator, the pigment production scores were 4+ for all media, but GBS colonies were smaller in the media prepared without serum (1- versus 2-mm diameters). Nevertheless, after 6 days at either 22 or 30°C, the media performed differently (Fig. 1). Media prepared with unmodified starches and serum supported good GBS pigment production (scores of 3+ and 4+). Media prepared with either 1 or 2% soluble starch and serum deteriorated, and pigment production diminished (scores of 1+ and 2+). All the media prepared without serum supported good GBS pigment production (scores of 3+ and 4+), but the colonies were smaller (about 1 mm in diameter). Owing to these results, and because the rice starch was easier to dissolve, the use of 1% rice starch plus 1% serum was chosen in place of 2% soluble starch plus 5% serum in the GM recipe.

To test the suitability of this modified GM to withstand stringent storage conditions, a field evaluation was carried out comparing GM prepared with 2% soluble starch and 5% horse serum (standard recipe), GM prepared with 2% soluble starch and 0% serum, and GM prepared with 1% unmodified rice starch and 1% serum. All the media were stored at 4°C either as they were prepared or after being kept for 6 days at 25°C.

Vaginorectal swabs ($n = 350$) from pregnant women were placed in tubes with 0.8 ml of 0.85% NaCl and swirled vigorously. Six additional swabs were immersed in a tube and used to inoculate one plate of the media assayed. The results are shown in Table 1. GBS recoveries (51 colonies from 350 swabs) from the following media were identical: (i) GM prepared with soluble starch and serum and then refrigerated as prepared, (ii) GM prepared with unmodified starch and serum and then refrigerated as prepared, and (iii) GM prepared with unmodified starch and serum and refrigerated after being kept for 6 days at 25°C. Nonetheless, GBS were recovered from 46 swabs in GM prepared with soluble starch and without serum and refrigerated either as the media were prepared or after being kept for 6 days at 25°C ($P < 0.05$, binomial test) and from 35 swabs in GM prepared with soluble starch and 5% serum and kept for 6 days at 25°C before being stored in the refrigerator ($P < 0.01$, binomial test). There was a slight loss of pigment intensity in the medium prepared with unmodified starch and serum, kept at 25°C for 6 days, and refrigerated afterwards. This loss of pigment did not impair the detection of any GBS-positive samples. There was an important loss of pigment in GM prepared with soluble starch and serum and stored for 6

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FIG. 1. GBS colonies (ATCC 12386) in GM stored for 6 days at 22°C. Left, GM prepared with 1% rice starch and 1% serum; center, GM prepared with 2% soluble starch but without serum; right, GM prepared with 2% soluble starch plus 5% serum.

days at 25°C before being refrigerated. GBS colonies were always smaller in the media without serum.

An important factor in the poor performance of nonrefrigerated GM prepared with soluble starch and serum is the hydrolysis of starch caused by α-amylase (16). This starch hydrolysis can be observed when plates of GM prepared with soluble starch and serum are left for a few days at room temperature, a drop of Lugol's solution is added, and the blue color of the iodine-starch complex does not appear. When GM is prepared with unmodified starch, the hydrolysis process takes longer, and the Lugol's solution produces the characteristic deep-blue color even if GM is kept for several days at 30°C. Moreover, α-amylase hydrolysis of starch produces maltose, from which GBS produces acid (19). This acid can overpower the buffering capacity of the medium and hinder GBS pigment production, which is impaired at pH values below 7 (4, 6, 13, 21). The starch hydrolysis is related to the amylase content of the serum used, and this can help explain the differences in stability observed among different batches of GM (14).

TABLE 1. Comparison of GBS culture results obtained from 350 vaginorectal swabs

GM formulation and storage conditions	No. of cultures					Colony diam (mm)
	With GBS detected	With pigment score[a] of:				
		4+	3+	2+	1+	
2% soluble starch, 5% horse serum						
Refrigerated as prepared	51	38	10	3	0	1.5–2
Refrigerated after 6 days at 25°C	35	0	6	19	10	1.5–2
2% soluble starch, without horse serum						
Refrigerated as prepared	46	32	8	6	0	1–1.5
Refrigerated after 6 days at 25°C	46	28	9	7	2	1–1.5
1% unmodified corn starch, 1% horse serum						
Refrigerated as prepared	51	38	10	3	0	1.5–2
Refrigerated after 6 days at 25°C	51	32	10	9	0	1.5–2

[a] See text for details.

A possible way of increasing GM stability is the elimination of serum (and amylase) from its formulation. Our data, however, support the observations of Noble et al. (13) indicating the superiority of serum-containing media to detect GBS pigment. The data also show that GM spoilage at room temperature is slower when there is no serum in the medium, but GBS colonies are smaller and pigment production is weaker. The increase in GBS pigment in media which contains both serum and starch (10, 13) might be explained by the need for starch for rapid pigment production (12) and by the facilitation of pigment production by maltooligosacharides produced during the starch hydrolysis (16). Nevertheless, when the starch is completely hydrolyzed, pigment production is weak (2). Nonetheless, a comprehensive explanation as to why pigment production is triggered in GM and why nonhydrolyzed starch (2, 6) is required for optimal pigment detection is not yet possible. The chemical structure of the pigment and the biochemical pathways involved in its production are unknown.

This work shows that GM stability improves with the use of 1% unmodified rice starch (Sigma 7260) instead of 2% soluble starch and with the use of 1% instead of 5% horse serum. These modifications can help in a safer use of GM to detect GBS.

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